

GROWTH-FACTOR-DEPENDENT STRAINS OF SALMONELLAE

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Most strains of *Salmonellae* have simple nutritional requirements and grow well, therefore, in simple media consisting of glucose, inorganic nitrogen, and mineral salts (Kauffmann, 1954). There are, however, a number of *Salmonellae* which are unable to synthesize certain essential vitamins and amino acids and these must be supplied in the culture medium. The need of *Salmonella typhosa* for tryptophan (Fildes *et al.*, 1933), of *Salmonella gallinarum* for vitamin B₁, and of *Salmonella pullorum* for nicotinic acid, leucine, aspartic acid, cystine, and other amino acids (Johnson and Rettger, 1943; Gilfillan *et al.*, 1955) has been described. Also, Lederberg (1947) has reported strains of *Salmonella paratyphi* A, *S. paratyphi* B, *S. choleraesuis*, *S. enteritidis*, *S. dublin*, and *S. typhimurium* which require for growth different combinations of vitamin B₁, biotin, and a variety of amino acids. Different strains of the same serotype may or may not require these growth factors.

In the course of our investigations of *Salmonellae* which develop slowly on ordinary culture media (Stokes and Bayne, 1957, 1958) we have had occasion to determine the specific growth factor requirements of a number of nutritionally dependent strains of *Salmonella typhisuis*, *S. abortusovis*, *S. fulica*, *S. paratyphi* A, *S. pullorum*, and *S. gallinarum*. The results which are reported in the present paper extend the list of growth factor dependent types of *Salmonellae* and disclose the existence of types with more complex requirements than any previously described.

MATERIALS AND METHODS

The *Salmonellae* investigated included 1 strain each of *S. fulica* and *S. paratyphi* A, 2 strains each of *S. typhisuis* and *S. abortusovis*, 6 strains of *S. pullorum*, and 12 strains of *S. gallinarum*. The cultures marked with the letter K were obtained from Dr. F. Kauffmann and those marked

with E from Dr. P. R. Edwards. We are indebted to them for these and additional strains. The stock cultures were maintained on trypticase soy agar slants.

The chemically defined basal medium consisted of the following ingredients: glucose, 1.0 per cent; (NH₄)₂SO₄, 0.1 per cent; Na citrate·2H₂O, 0.05 per cent; MgSO₄·7H₂O, 0.05 per cent; phosphate buffer, pH 7.1, 0.05 M; and distilled water. In our experience, the citrate is necessary for the growth of strains which do not require an exogenous supply of growth factors. The glucose was autoclaved separately and added aseptically to the medium after the latter had been prepared, distributed in 5 ml amounts in test tubes, and autoclaved.

When it was necessary to enrich the basal medium with a mixture of amino acids, 0.3 per cent vitamin-free casein hydrolyzate was added and also 25 µg of L-cystine and 100 µg of DL-tryptophan per ml of medium. To determine specific amino acid requirements, the casein hydrolyzate was replaced by a mixture of 20 amino acids. The latter included the DL isomers of leucine, isoleucine, valine, methionine, tryptophan, phenylalanine, glutamic and aspartic acids, alanine, threonine, serine, and norleucine; the L-isomers of cystine, tyrosine, lysine, arginine, histidine, proline, and hydroxyproline; and also glycine. Each amino acid was used in a concentration of 0.2 mg per ml of medium except for 0.4 mg of tryptophan and 0.1 mg of lysine.

The B-vitamins employed and their final concentrations per ml of medium were: thiamin, riboflavin, nicotinic acid, and pantothenic acid, 0.2 µg each; pyridoxin, 1.0 µg; *p*-aminobenzoic acid, 0.4 µg; folic acid, 0.01 µg; and biotin, 0.0002 µg. Purines and pyrimidine were supplied usually as a mixture of adenine, guanine, and uracil and at a concentration of 10 µg of each per ml of medium.

The details of the preparation of the stock solutions of the amino acids, vitamins and purines, and pyrimidine have been given previously (Stokes and Gunness, 1945).

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Inocula were prepared by transferring, with a needle, a small amount of growth from agar slant cultures of *Salmonellae* to 8 ml of sterile distilled water in tubes. One drop of the resulting faintly turbid suspensions was used to inoculate each tube of experimental medium. All experiments were incubated at 35 C. Growth was either estimated visually or measured quantitatively in a Klett-Summerson photometer (red filter). Additional technical details will be given in the text as required.

RESULTS

Most of the experiments involved the E and K strains of *S. typhisuis* and *S. abortusovis*, and also *S. fulica* and *S. paratyphi* A. None of these 6 strains grew in the chemically defined basal medium composed of glucose, inorganic nitrogen, and mineral salts. The effect of the addition of vitamins, purines and pyrimidine, and amino acids (casein hydrolyzate) as groups and in all possible combinations was determined. All strains grew in the presence of appropriate combinations of growth factors. The requirements for the individual strains, in order of increasing complexity are:

- S. fulica*—amino acids
- S. paratyphi* A—amino acids, purines-pyrimidine
- S. abortusovis* (E)—amino acids, vitamins
- S. typhisuis* (K)—amino acids, vitamins
- S. abortusovis* (K)—amino acids, vitamins, purines-pyrimidine
- S. typhisuis* (E)—amino acids, vitamins, purines-pyrimidine

Although *S. fulica* needed only amino acids, the addition of vitamins greatly stimulated growth. The requirement of *S. paratyphi* A, *S. abortusovis* (K), and *S. typhisuis* (E) for the purine-pyrimidine mixture was unexpected since to our knowledge, such naturally occurring strains have not been reported previously in the literature. Moreover, the requirement of *S. abortusovis* (K) and *S. typhisuis* (E) for a combination of amino acids, vitamins, and the purine-pyrimidine mixture, sets these two strains apart as being more complex in their nutritional requirements than other growth-factor-dependent strains of *Salmonellae*. In this respect the two strains resemble many of the lactic acid bacteria which require similar combinations of growth factors.

Each group of growth factors was investigated

to determine the specific one required for the growth of the individual strains of *Salmonellae*.

Purines. The basal medium enriched with casein hydrolyzate and the vitamin mixture was used to determine the specific purine requirements. Uracil was not essential for the growth of *S. paratyphi* A, *S. abortusovis* (K), and *S. typhisuis* (E). These strains, therefore, need only purines. Guanine alone permitted rapid growth of *S. paratyphi* A, whereas growth with adenine alone was delayed for a day. This situation was reversed with the other two strains, best growth being obtained with adenine. The interchangeability of guanine and adenine suggests that the three *Salmonellae* can convert one purine into the other as has been shown for *Lactobacillus casei* (Baylis *et al.*, 1951).

Other purines and derivatives were tested at a level of 10 μ g per ml of medium as substitutes for guanine and adenine. Growth of all three strains was rapid with hypoxanthine and somewhat delayed with xanthine. Uric acid and allantoin were completely inactive. The nucleosides, guanosine, and adenosine, were less effective than the corresponding free purines. Thus *S. typhisuis* (E) failed to grow with guanosine and growth of *S. paratyphi* A with adenosine was delayed for several days. Inosine, however, was as effective for all three strains as hypoxanthine. Also, the nucleotides of the purines were less effective than the free purines. *S. typhisuis* (E) could not grow with guanosine-5'-PO₄ and the growth of the other two strains was delayed. *S. paratyphi* A did not develop with adenosine-5'-PO₄ and the growth of *S. typhisuis* (E) was delayed. All strains exhibited slow growth with inosine-5'-PO₄ which is in contrast to the rapid growth with the free base, hypoxanthine, and the corresponding nucleoside, inosine.

Therefore, in general, the purine bases are more active than their nucleosides and the latter, in turn, are more active than the corresponding nucleotides. This sequence of activity may indicate that the cells must convert the nucleosides and nucleotides to the free bases to permit entry into the cells or for subsequent internal use in the synthesis of nucleic acids. This conversion may take place slowly or not at all (Lawrence, 1955; Aaronson and Rodriguez, 1957).

Quantitative determinations established that 10 μ g of adenine or guanine per ml of medium is necessary for maximum growth of the *Salmonellae*.

Vitamins. For four of the six strains, vitamins are essential for growth. The basal medium enriched with casein hydrolyzate and the purine-pyrimidine mixture was used to determine the essential vitamins in the mixture of eight originally employed. Only thiamin and nicotinic acid are essential for the four strains. *S. abortusovis* (K) needs thiamin and *S. abortusovis* (E) needs nicotinic acid. In contrast, *S. typhisuis* (K) requires both thiamin and nicotinic acid. Also *S. typhisuis* (E) requires both vitamins for rapid growth but can develop without nicotinic acid on incubation for several days. Whether the delayed growth is due to slow synthesis of nicotinic acid or to the development of a nicotinic acid independent mutant was not determined.

All strains could be serially subcultured several times in the presence of only the essential vitamins. This eliminates the possibility that additional vitamins are required which might have been introduced into the media by the original inocula. It is of interest that thiamin and nicotinic acid are the vitamins most commonly required by growth factor dependent strains of Salmonellae and only rarely does a need for some other vitamin appear.

The amounts of thiamin and nicotinic acid, per ml of medium, necessary for maximum growth are 0.003 μ g of thiamin for *S. abortusovis* (K), 0.08 μ g of nicotinic acid for *S. abortusovis* (E), 0.003 μ g of thiamin and 0.1 μ g of nicotinic acid for *S. typhisuis* (K), and 0.008 μ g of thiamin for *S. typhisuis* (E) and also 0.03 μ g of nicotinic acid for rapid growth. These quantities are similar to those required by other thiamin and nicotinic acid dependent bacteria (Stokes *et al.*, 1944).

Amino acids. As indicated previously, all six strains require casein hydrolyzate for growth. To determine the essential amino acids for each strain, the casein hydrolyzate was replaced with a mixture of 20 amino acids. The complete B-vitamin and purine-pyrimidine mixtures were in the basal medium. All strains grew well with the 20 amino acids. Specific requirements were determined by omitting 1 amino acid at a time from the mixture of 20 and observing the effect on growth. The results of these experiments disclosed the essential and stimulatory amino acids for each strain.

For *S. typhisuis* (E), cystine, leucine, and threonine are indispensable, and phenylalanine and histidine are stimulatory. In contrast, *S. typhisuis* (K) requires only cystine. For *S. abortusovis* (E)

cystine and methionine are essential and valine and threonine are markedly stimulatory. *S. abortusovis* (K) requires cystine and leucine and is stimulated by threonine. Cystine and arginine are essential for *S. paratyphi* A. *S. fulica* is unusual in that although it requires amino acids for growth, omission of any 1 amino acid from the mixture of 20 did not prevent growth. These results indicate that there are at least 2 amino acids in the mixture which can, interchangeably, supply the needs of *S. fulica* so that when 1 amino acid is omitted, the other supports growth. This was found to be true. When each of the 20 amino acids is added singly to the basal medium, cystine or histidine permits good growth of *S. fulica*. It is doubtful that the activity of histidine is due to contamination with cystine since three different samples of L-histidine were equally active.

As a further check on the essentiality of the amino acids as determined in the previous elimination type of experiment, an attempt was made to grow the six strains with only the indispensable amino acids present. All strains except *S. abortusovis* (E) and *S. typhisuis* (E) grew well with only the essential amino acids. For *S. abortusovis* (E) growth occurred only when a combination of the essential and stimulatory amino acids were added to the basal medium. *S. typhisuis* (E) not only required the essential stimulatory amino acids but also an additional source of organic nitrogen for growth. Asparagine, 0.1 per cent, was satisfactory for this purpose. Asparagine also stimulated the growth of some of the other strains. It should be emphasized that growth in the minimal amino acid media was considerably slower than in media containing casein hydrolyzate. Maximum growth occurred with the latter generally in one day whereas 2 to 3 days were usually required for good growth in the minimal media.

The complete growth factor requirements of the six Salmonellae are shown in table 1.

S. pullorum and *S. gallinarum*. Six strains of *S. pullorum* were examined for their growth factor requirements. None of the strains grew in the basal medium but multiplied readily when casein hydrolyzate was added. None of the strains require B-vitamins or the purine-pyrimidine mixture. Analyses of their amino acid requirements showed that cystine and leucine are essential for four strains and that these two amino acids plus aspartic acid are required by the re-

TABLE 1
Growth factor requirements of some strains of *Salmonella*

Strains	Purines	Vitamins	Amino Acids	
			Essential	Stimulatory
<i>Salmonella typhisuis</i> (E)	Adenine	Thiamin Nicotinic acid*	Cystine Leucine Threonine	Histidine Phenylalanine
<i>Salmonella typhisuis</i> (K)		Thiamin Nicotinic acid	Cystine	
<i>Salmonella abortusovis</i> (E)		Nicotinic acid	Cystine Methionine	Valine Threonine
<i>Salmonella abortusovis</i> (K)	Adenine	Thiamin	Cystine Leucine	Threonine
<i>Salmonella fulica</i>			Cystine or histidine	
<i>Salmonella paratyphi</i> A	Guanine		Cystine Arginine	

* cf. text.

maining two strains. Arginine and isoleucine are stimulatory for all strains. All strains grew in the basal medium to which the above amino acids were added. In general, the results are similar to those obtained by other investigators for other strains of *S. pullorum*. Johnson and Rettger (1943), however, did not include data on cystine in their extensive survey. This amino acid is almost invariably required by strains of *S. pullorum* and other growth factor dependent strains of *Salmonellae*.

Likewise, out of 12 strains of *S. gallinarum* which did not grow in the basal medium, 10 strains developed when thiamin was added. However, growth is very slow and a week is needed for appreciable development. In contrast, full growth occurs in 1 day with casein hydrolyzate or a mixture of cystine, leucine, and aspartic acid. Amino acids, therefore, are markedly stimulatory and almost essential. Of the 2 strains which did not grow in the basal medium containing thiamin, 1 strain grew with amino acids but the other strain did not. The additional growth factors required by this last strain were not determined. The essentiality of thiamin for our strains of *S. gallinarum* is in agreement with the data of Johnson and Rettger for 22 strains of *S. gallinarum*, all of which required thiamin. The

latter investigators did not attempt to grow their strains without amino acids and reported that 5 out of 10 strains required leucine. The slow but definite growth, therefore, of almost all our strains in the absence of any amino acids is noteworthy.

DISCUSSION

Our results and those of other investigators indicate that a considerable number of naturally occurring strains of *Salmonellae* are unable to synthesize essential vitamins, amino acids, and purines. This biochemical deficiency in any one strain may encompass one or all of the three categories of growth factors. The latter must be supplied in culture media for growth of the nutritionally dependent strains of *Salmonellae*. This aspect is important in the isolation of such strains from natural habitats. Some of the customary bacteriological media may not contain adequate amounts of thiamin and other vitamins (Stokes *et al.*, 1944). Also, cystine, which is an essential requirement for most growth-factor-dependent strains, may be easily destroyed or made unavailable during the preparation of culture media (Lankford *et al.*, 1957). Consideration should be given, therefore, to the nutritional adequacy of particular media used to isolate *Salmonellae*.

from natural sources and to enrichment of such media with mixtures of vitamins, cystine, and other known growth factors to provide the essential needs of nutritionally dependent strains.

SUMMARY

The growth factor requirements of strains of *Salmonella typhimurium*, *Salmonella abortusovis*, *Salmonella fulica*, *Salmonella paratyphi* A, *Salmonella pullorum*, and *Salmonella gallinarum* include the vitamins thiamin and nicotinic acid, the purines adenine and guanine, and the amino acids cystine, methionine, leucine, threonine, histidine, arginine, and aspartic acid.

The particular combination of these growth factors essential for each strain has been determined and also the quantities of the vitamins and purines which are necessary for maximum growth. The growth-factor requirement of a particular *Salmonella* strain may vary from a single vitamin or amino acid to a complex of two vitamins, a purine, and several amino acids. The various purines are interchangeable to a considerable extent and the free bases are more active than the corresponding nucleosides or nucleotides.

The significance of growth factor requirements for the isolation of members of the genus *Salmonella* is discussed.

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